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Research Article

Comparing sampling protocols and results to better detect the amphibian fungal parasite *Batrachochytrium dendrobatidis* in the wild

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Abstract

The pathogenic fungus *Batrachochytrium dendrobatidis* (Bd) causes chytridiomycosis in amphibians across the world, increasing the risk of population declines and species extinctions. Using a traditional sampling approach with traps, great crested newts (*Triturus cristatus*) and smooth newts (*Lissotriton vulgaris*) have been sampled in eight ponds for population estimates since 2013. The presence of Bd has been investigated by environmental DNA (eDNA) in the ponds since 2017, but results have often varied between sampling methods used during the same sampling period. By comparing results from pond water using two filter pore sizes (0.45 and 2.0 µm) and from amphibians (filtered bathwater, soft skin swabs, soft sandpaper rubbed on skin, and skin samples from between toes from toad carcasses), the results showed that filtered bathwater samples or gently rubbing amphibian skin with soft sandpaper are the most reliable methods for obtaining Bd DNA. Results from pond water filtered through the two pore sizes did not differ significantly across positive sites. Results also indicated that a variety of DNA concentrations should be tested in replicated qPCRs or ddPCRs to account for both potential inhibition and low levels of Bd DNA in the samples. Both crested newts and smooth newts were identified as infected with varying prevalence, but no trends in population declines were observed for any of the species during the sampling years 2013–2024. However, the number of crested newts in the monitoring ponds was altogether low and sometimes sporadic, and ponds with higher numbers of crested newts should be included in future studies on population trends in infected populations.

Key words: Amphibians, Bd, eDNA, false negatives, method development, species detection

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Introduction

Invasive alien species are regarded as one of the most important threats to native biodiversity because they can lead to local extinctions and the collapse of habitat types (Capinha et al. 2015; Early et al. 2016; Strand et al. 2019; Pysek et al. 2020). Invasive alien species are, however, not always easy to detect: most would recognize an elephant in the Arctic as an animal that does not belong there, but few, if any, would spot invasive microorganisms living in their garden – even though it is highly likely that some have already moved in (Lovett et al. 2016; Scott-Brown et al. 2018). The smaller the alien species, the less likely it is to be perceived by humans, but accurate recognition of pathogens is critical for wildlife disease research and conservation (Mörner et al. 2002; Buttke et al. 2015; Barroso et al. 2021; West et al. 2024).

Amphibians have been declining throughout the world since at least the 1950s (Houlahan et al. 2000; Finn et al. 2023), where the reasons for their disappearance are interactive and complex (Blaustein and Kiesecker 2002; Reid et al. 2019; Mi et al. 2023). Diseases caused by invasive alien pathogens are one of the factors driving these ever-lower numbers (Fisher et al. 2012; Martel et al. 2014; Fisher and Garner 2020; West et al. 2024). Invisible to the human eye, the generalist fungal amphibian skin pathogen *Batrachochytrium dendrobatidis* (hereafter referred to as Bd) (Longcore et al. 1999) has spread from its natural range in Asia (O'Hanlon et al. 2018; Sun et al. 2025) to every continent inhabited by amphibians (Greener et al. 2020), most likely through the pet and plant trade (Fu and Waldman 2022; Laorden-Romero et al. 2024). Bd was first discovered in Europe following a mass mortality event in 1997 (Bosch et al. 2001) and is now present in most European countries (Garner et al. 2005; Allain and Duffus 2019). In Norway, Bd was first detected from environmental DNA (eDNA) in 2017 from water collected from newt ponds (Taugbøl et al. 2021).

Species detection from genetic traces in the environment is increasingly used in routine species surveys (Deiner et al. 2017; Fediajevaite et al. 2021; Sandercock et al. 2023) and is also a commonly used non-invasive method for detecting Bd (Chestnut et al. 2014; Brannelly et al. 2020a; Taugbøl et al. 2021; Everts et al. 2025). However, the application of eDNA species detection is not straightforward, and sampling from wild environments has a high chance of producing false negatives (Ficetola et al. 2015; Congram et al. 2022; Nogueira et al. 2025; Taugbøl et al. 2025). Bd lives as a sporangium in the keratinized skin of amphibians and spreads via aquatic zoospores that swim for about 2 cm (Piotrowski et al. 2004). Therefore, when targeting only water samples, the likelihood of detecting Bd DNA will depend on various factors, such as collecting water near infected hosts that are actively shedding the pathogen, the average infection load of hosts (Longo et al. 2023; Hartmann et al. 2024), and the quantity of zoospore release at the time of sampling. Swabbing has been the recommended (Hyatt et al. 2007) and most commonly used (Shin et al. 2014) sampling protocol for Bd since 2007. However, studies have since found the method to yield inconsistent results, especially for animals or sites with low infection intensity (Shin et al. 2014). For species negatively affected by Bd infection, valid inference of infection status is key to enabling rapid responses to prevent further pathogen pollution.

Little is known about how Bd spreads from site to site once locally introduced (Johnson and Speare 2005; Liew et al. 2017; Prado et al. 2023) or what effect the parasite has once established, as not all amphibians respond equally to infection (Schloegel et al. 2006; Ryan et al. 2008; Crawford-Ash and Rowley 2021; Harmos et al. 2021). One method to measure potential effects of Bd in the wild is to collect data on both Bd-infected and Bd-free populations within the same geographic area (Pilliod et al. 2010; Taugbøl et al. 2021; West et al. 2024). The primary comparison between infected and non-infected ponds in Norway in 2017 concluded that there was little evidence for population decline of both newt species in Bd-infected ponds, but this finding may have been due to the inclusion of false-negative ponds in the "non-infected" group, thereby basing the model on incorrect input data. Furthermore, there were no data on how long Bd had been present in the ponds, which species were infected, or the prevalence among the newts occupying the Bd-infected ponds (Taugbøl et al. 2021). Further, the infected Norwegian ponds were scattered and intermingled with Bd-free ponds, and although this is com-

monly observed in other survey areas (Kärvemo et al. 2018; Congram et al. 2022), suboptimal Bd detection methods could result in false negatives and an overall underestimation of the distribution range. When the Bd-positive Norwegian ponds were resampled in 2018, only one of the five ponds previously found to be infected in 2017 tested positive through filtered water samples (with more than two-thirds of qPCRs testing positive). Negative Bd results from three of the ponds conflicted with Bd-positive swabs collected from the same ponds, strengthening the assumption that the 2017 dataset included false negatives that may have confounded the population modeling results.

This study compares how Bd detection varies with a range of sampling methods – including filtered water samples from pond water and amphibian bathwater and swabs from newts and toads (*Bufo bufo*) – from wild populations in southeastern Norway. The main objective was to identify a cost-effective sampling strategy that minimizes false negatives to the extent possible. As the funding sources and goals have varied across years, the direct comparisons across methods are kept within species and sampling years when more than one sample type was collected.

Materials and methods

Study area and limitations to sampling

Sampling was conducted in the southeastern part of Norway (Fig. 1a) in two areas where Bd had previously been detected (Taugbøl et al. 2021; Strand et al. 2025). Newt samples were collected from eight ponds south of Oslo. Pond water was filtered in all sampling years for most ponds, as one of the original main aims was to check whether ponds with larger populations of newts also had higher levels of newt eDNA and if eDNA could thereby substitute monitoring with traditional trapping (Taugbøl et al. 2025). The same DNA extracts were also routinely tested for Bd, but as some ponds varied in infection status within and across years, additional alternative samples were needed to better determine the infection status of the ponds. Due to limited annual funding, comparable samples within a year were collected only during three of the eight sampling years from newt ponds: 2018, 2023, and 2024.

Toad samples were collected from carcasses found at a road crossing in Nittedal (Fig. 1b). Obtaining sampling permits for tissue collection from live anurans (e.g., toes) is increasingly difficult. The original objective of the toad samples presented here was to assess toad DNA quality across several external sampling methodologies and to test whether the same samples could also yield reliable results for individual Bd infection status (Taugbøl 2024). As DNA from tissue would represent the relative DNA quality against which alternative samples were compared, all samples were collected from recently overrun toads from Nittedal, from a toad population found to have a Bd infection prevalence of 80% (Strand et al. 2025).

Water filtration protocol from newt ponds

Pond water samples were collected prior to trap placement during all sampling years. From each pond, 15 subsamples (collected 1–4 m apart) of 0.2 L surface-sampled water were pooled into a mixed sample (Fig. 2a). Subsampling increases the likelihood of filtering a sample that more closely represents the pond as a whole, as eDNA is likely to be unevenly distributed (Troth et al. 2021; Congram et al. 2022;

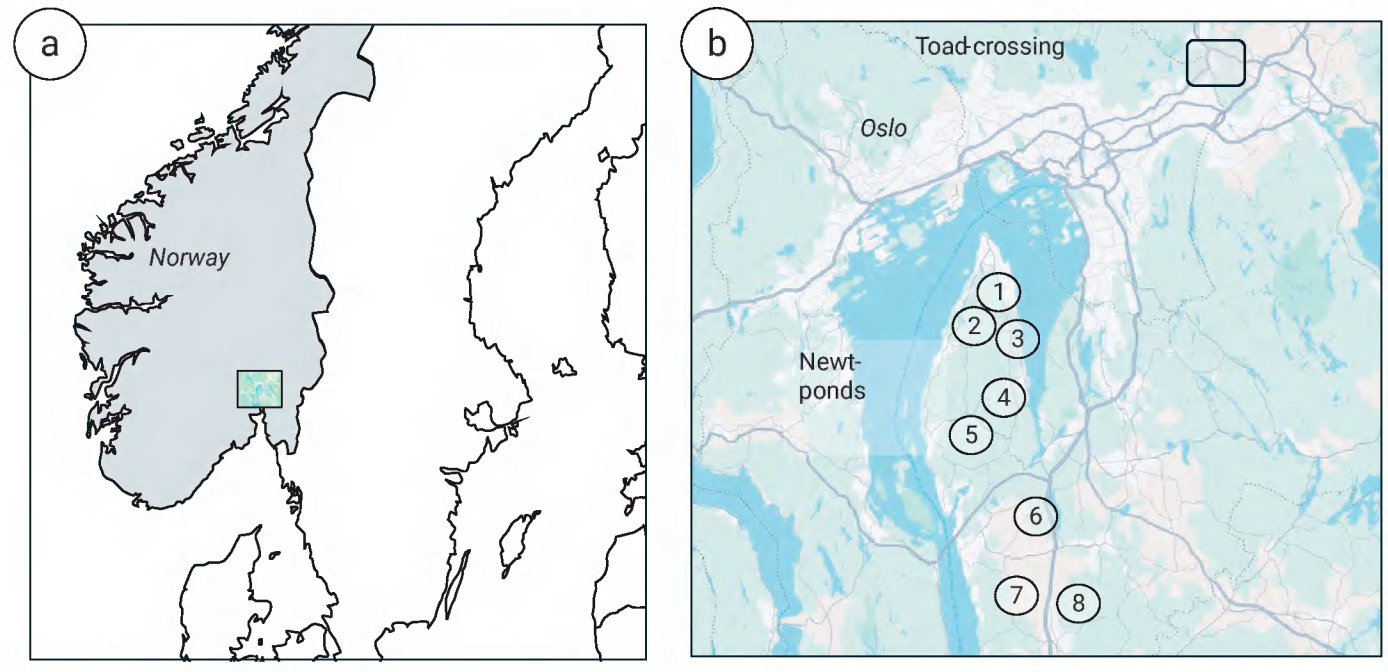

Figure 1. Sampling sites. **a.** The positions of the sampling sites in the southeastern part of Norway are shown as a green square; **b.** The sampling locations close to the capital of Norway (Oslo), where the square marked "toad crossing" indicates the site where the toad samples were collected, and the encircled numbers in the south indicate newt ponds: (1) Røer gård, (2) Garderenga, (3) Østre Glenne, (4) Ottarsrud, (5) Bellsjødammen, (6) Tokerud, (7) Solberg, and (8) Østre Støkken. Both maps were created with ArcGIS (Enterprise 11, 2022) in combination with Adobe Illustrator (Adobe Inc. 2019).

Taugbøl et al. 2025). Due to high repeatability in eDNA results from newts filtered from the same mixed water source in 2018 (Taugbøl et al. 2025), only one sample of 0.5 L was filtered through a 0.45 µm cellulose nitrate filter (Fig. 2a; Nalgene CN 145-0045, Thermo Scientific) using a vacuum pump (Sartorius Microsart e.jet) connected to a three-place manifold (Pall filter manifold) in 2018. After filtration, each filter was preserved in a 2 mL tube containing 1440 µL ATL buffer (Qiagen). Based on previous findings of seasonal increases in Bd eDNA in one pond identified as positive in 2017 (Taugbøl et al. 2021), pond water was sampled and filtered four to five times from each pond in 2018: May 12 and 23, June 8, and July 3 and 13. Additionally, for a subset of ponds and sampling times in 2018 – as well as during later sampling years – water from the mixed sample was filtered through 2.0 µm glass fiber filters (Merck Millipore; Fig. 2a) using a self-priming peristaltic pump. These larger pore-size filters allowed filtration of a greater water volume (approximately 1–1.5 L) and were expected to increase the likelihood of detecting Bd DNA (Fossøy et al. 2020). In 2024, mixed pond water was filtered through duplicate sets of 0.45 µm and 2.0 µm filters during three time points in May: May 13–14, 22–23, and 27–28. For all sampling years, negative controls consisting of lake water and/or tap water filtered in the field were included to assess contamination risks (0.45 µm filters in 2018; 2.0 µm glass fiber filters in all other sampling years).

Sampling newts with traps, bathwater, and soft swabs

Great crested newts and smooth newts were caught using funnel traps originally designed for minnows. For each pond, 10 traps were systematically set along the shoreline during the peak breeding season each year and left in place for approximately 24 hours. Captured newts were placed in clean containers, unpacked at the site, and filled with pond water. In 2023 and 2024, 1–1.5 L of newt bathwater was filtered through a 2.0 µm glass fiber filter (Fig. 2b; Merck Millipore) using a self-priming peristaltic pump. In 2018, 2020, and 2023, newts were individually

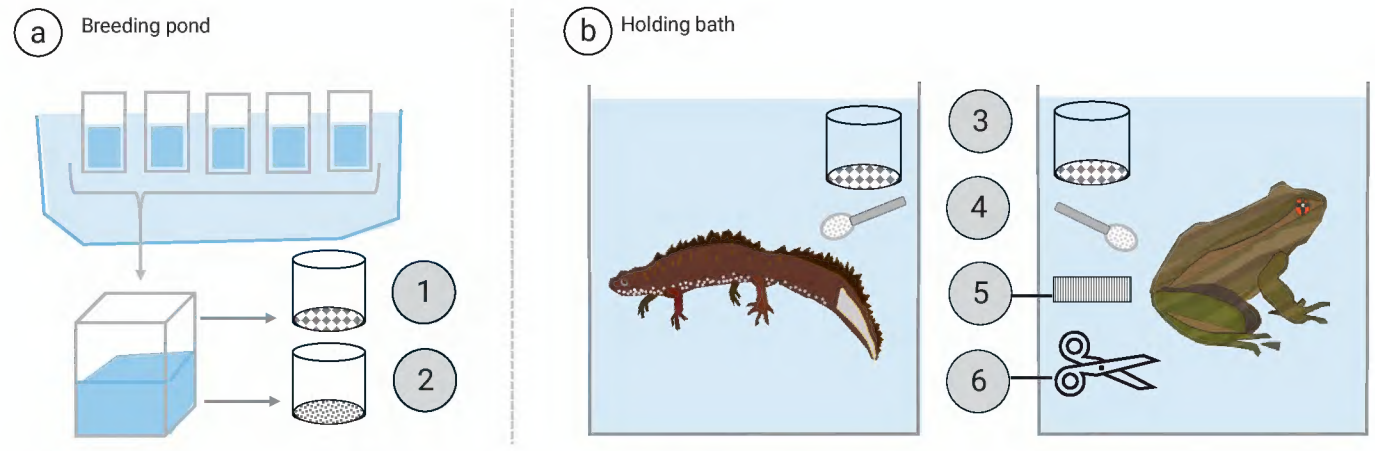

Figure 2. Overview of samples. **a.** Water filtering: water was collected from several sites within each pond and mixed before being filtered through either (1) a 0.45 µm filter or (2) a 2.0 µm filter; **b.** Amphibian samples: (3) filtered bathwater filters pond water containing amphibians for 10–15 minutes (2.0 µL); (4) soft skin swabs; (5) toads only, soft sandpaper; (6) toads only, skin sample from between the two longest toes of one hind leg. All sampled toads were roadkill casualties. Illustrations were created using PowerPoint (Microsoft) and Adobe Illustrator (Adobe Inc. 2019).

selected and swabbed repeatedly over the feet and mouth with a soft cotton swab (Fig. 2b). All swab samples collected in 2018 and 2020 were stored individually in marked 1.5 mL Eppendorf tubes prefilled with 150 µL ATL buffer. In 2023, multiple swabs per pond (up to six) were stored in 5 mL Eppendorf tubes containing 4 mL ATL buffer as collective samples to test for Bd presence in five of the ponds. All captured individuals were released back into the ponds. For each pond, a catch per unit effort (CPUE) was calculated for each species as the number of animals divided by the product of trapping time and number of traps. Data on CPUE for the sampling years 2013–2016 were collected from Dervo et al. (2017).

Sampling toad carcasses

All toad samples were collected in April 2022. The toad sampling site (Fig. 1b) was chosen for two main reasons: first, toads from the site tested positive for Bd in 2021 (Strand et al. 2025); second, numerous roadkill casualties allowed for tissue sampling from carcasses as a comparative method for toad DNA. All observed live toads were carried across the road to the pond.

A total of six fresh carcasses with minimal external damage were used to collect the following samples (Fig. 2b): soft cotton swabs – each of two randomly selected feet was swabbed 10 times; soft sandpaper (P400) – the remaining two feet were each swabbed 10 times; swim skin – a piece of webbed skin between the two longest toes from one of the hind legs; and toes from carcasses. The swab, sandpaper, and skin samples were stored in 150 µL lysis buffer. Replicates of soft skin swabs were also stored dry in Eppendorf tubes before DNA extraction.

The bathwater sample was collected by filtering 0.5 L of pond water in which the carcass of each animal had spent 10–15 minutes (Fig. 2b). Filtered bathwater was always collected last to avoid influencing the results of the other sampling methods, in case exposure to water altered Bd detection. Toad bathwater was filtered through a 2.0 µm glass fiber filter with binder (Millipore) using a self-priming peristaltic pump (Makita USA Inc.) and Nalgene™ Single Use Analytical Filter Funnels (Thermo Scientific™). Each filter was cut in half, folded, and stored in two Eppendorf tubes prefilled with 1500 µL lysis buffer. All samples were kept at room temperature before being processed in the laboratory.

Molecular methods

DNA from the 0.45 µm filters collected from the newt ponds, as well as from all swabs collected from newts, was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the modified protocol of Spens et al. (2017). DNA attached to the 2.0 µm glass fiber filters was extracted using a modified NucleoSpin Plant II Midi (Macherey-Nagel) protocol. DNA from all toad samples was extracted using the salt-extraction method developed by Aljanabi and Martinez (1997).

After DNA extractions, the presence of Bd DNA was tested using duplicate digital droplet polymerase chain reactions (ddPCRs) in 2017 and 2018, with details as described in Taugbøl et al. (2021). Results were classified based on droplet counts: samples with three or more positive droplets were considered positive, while those with one to two droplets were considered weakly positive (Dobnik et al. 2015). Quantitative polymerase chain reaction (qPCR) was run in triplicate with species-specific primers for Bd, following the setup described by Boyle et al. (2004). qPCRs were conducted with 1 ng of input DNA across all years. Samples collected in 2024 (newts) and 2022 (toads) were also tested with 5 ng of input DNA. For all runs (ddPCR and qPCR), one or more negative controls were included as templates on each plate, using dH_2O and fish DNA as templates, along with a positive sample of Bd DNA. None of the negative field samples or technical controls amplified Bd DNA.

Statistical analysis

All analyses were performed in R 4.0.1 (R Development Core Team 2021). Differences in individual Bd concentrations for each sampling method were tested using two-tailed t-tests and General Linear Models (GLMs) implemented in base R. Visualization of results was plotted using a combination of base R graphics and ggplot2 (Wickham 2016). Plots were further combined using Adobe Illustrator (Adobe Inc. 2019). All data are available in Suppl.material 1.

Results

Detection of Bd from newt ponds in relation to CPUE

Trapping data (2013–2024) and Bd results (2017–2024, except 2021) are summarized for both newt species in Fig. 3. Apart from pond 8, no ponds tested consistently positive for Bd throughout the sampling years, and Bd results from mixed pond water filters and samples from bathwater and soft skin swabs were contradictory for ponds 1–4 and pond 7 for at least one sampling year (Fig. 3, heatmap). Due to the low number of ponds (n = 8) and the high likelihood of false-negative results, catch per unit effort (CPUE) for the newts is presented only as stacked bar plots (Fig. 3) and was not compared statistically based on infection status.

Bd detection from pond water and newt swabs in 2022

In 2018, both mixed pond water and individual soft skin swabs were collected from the five ponds identified as Bd-infected in 2017. Great crested newts were identified as positive in four of the ponds (Fig. 4a). The prevalence of Bd DNA from the

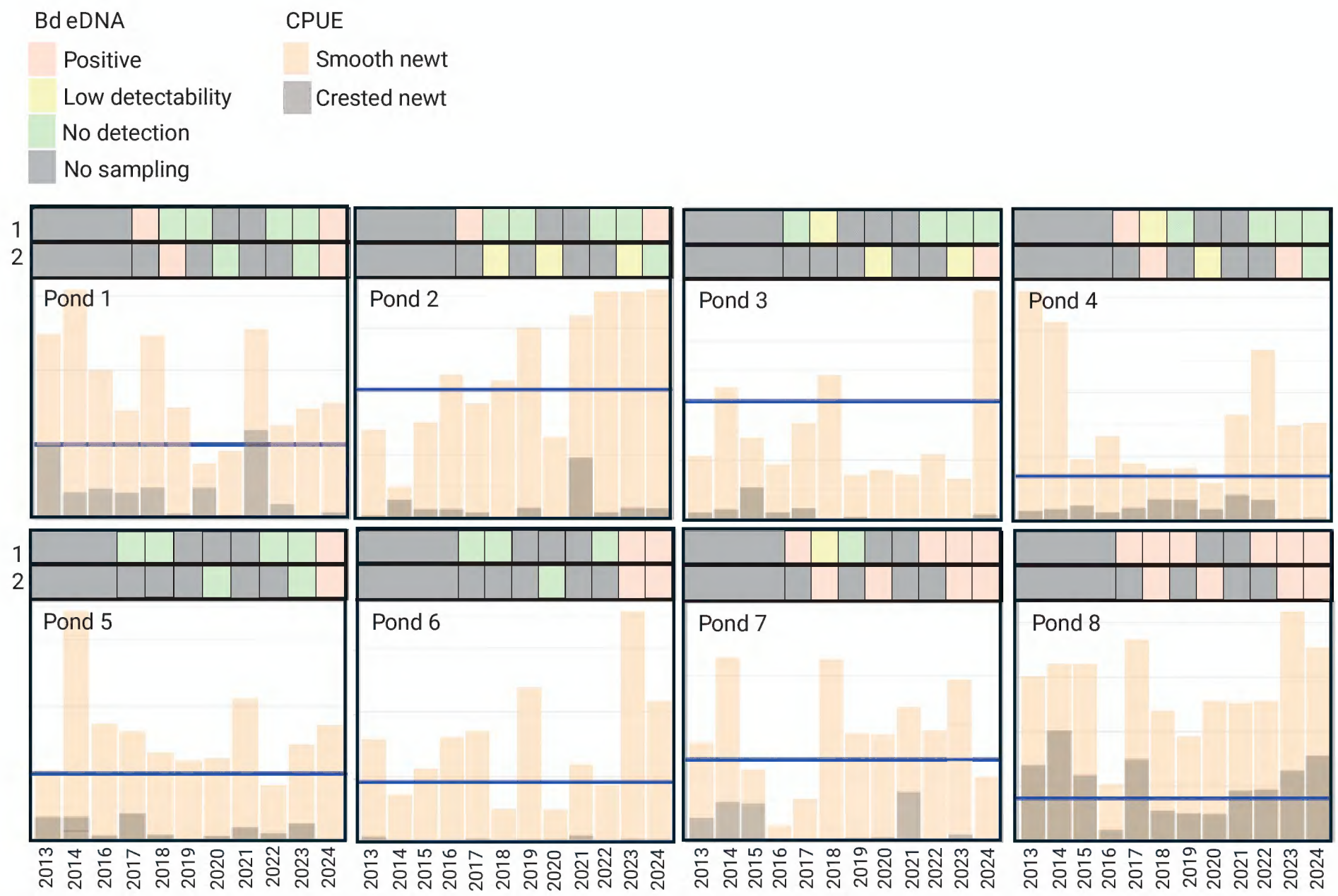

Figure 3. Bd infection status across years and estimates of newt populations per pond. The upper heatmap for each pond illustrates whether the pond was identified as infected (light pink), marginally infected (yellow; defined as fewer than three positive droplets in the ddPCRs or less than 66% positive qPCRs), or not infected by Bd (green), based on two sample types: (1) filters from mixed water samples and (2) samples collected from bathwater and/or soft skin swabs. The lower stacked bar plot illustrates catch per unit effort (CPUE) of smooth newt (light beige, upper part) and crested newt (light brown, lower part) for the eight ponds throughout the years 2013–2024. CPUE refers to the number of individuals caught in each pond, adjusted for trapping effort (number of traps × time in water). The horizontal blue line represents a CPUE of 0.2 to illustrate differences in population estimates between ponds.

individual swabs ranged from 50% infected crested newts at pond 8 (based on the three-droplet cutoff) to 90% in the same pond when including samples with one to two positive droplets (Fig. 4a). Using three or more droplets as a cutoff, only pond 1 had positive samples for smooth newts, whereas all five ponds had ddPCRs with one to two positive droplets for this species (Fig. 4b). Filters from pond water identified two ponds as positive with three or more positive droplets – pond 7 and pond 8 – whereas pond 4 was positive with one to two droplets (Fig. 4c).

Bd detection from pond water, newt swabs, and newt bathwater in 2023

A total of three sample types were collected and compared for Bd detection in 2023: mixed pond water (2.0 μm), newt bathwater (2.0 μm), and swab collections (more than one swab included in the same tube as a collective "pond sample," collected only from ponds 6, 7, and 8). In total, six of the ponds tested positive for Bd in one or more sample types (Fig. 3). Mixed pond water identified three ponds as positive (Fig. 5): pond 6 (first time detected as Bd positive, with five of six positive qPCRs), pond 7 (one of six qPCRs positive from 1 ng of DNA input), and pond 8 (three of six qPCRs positive, all at 1 ng of DNA input).

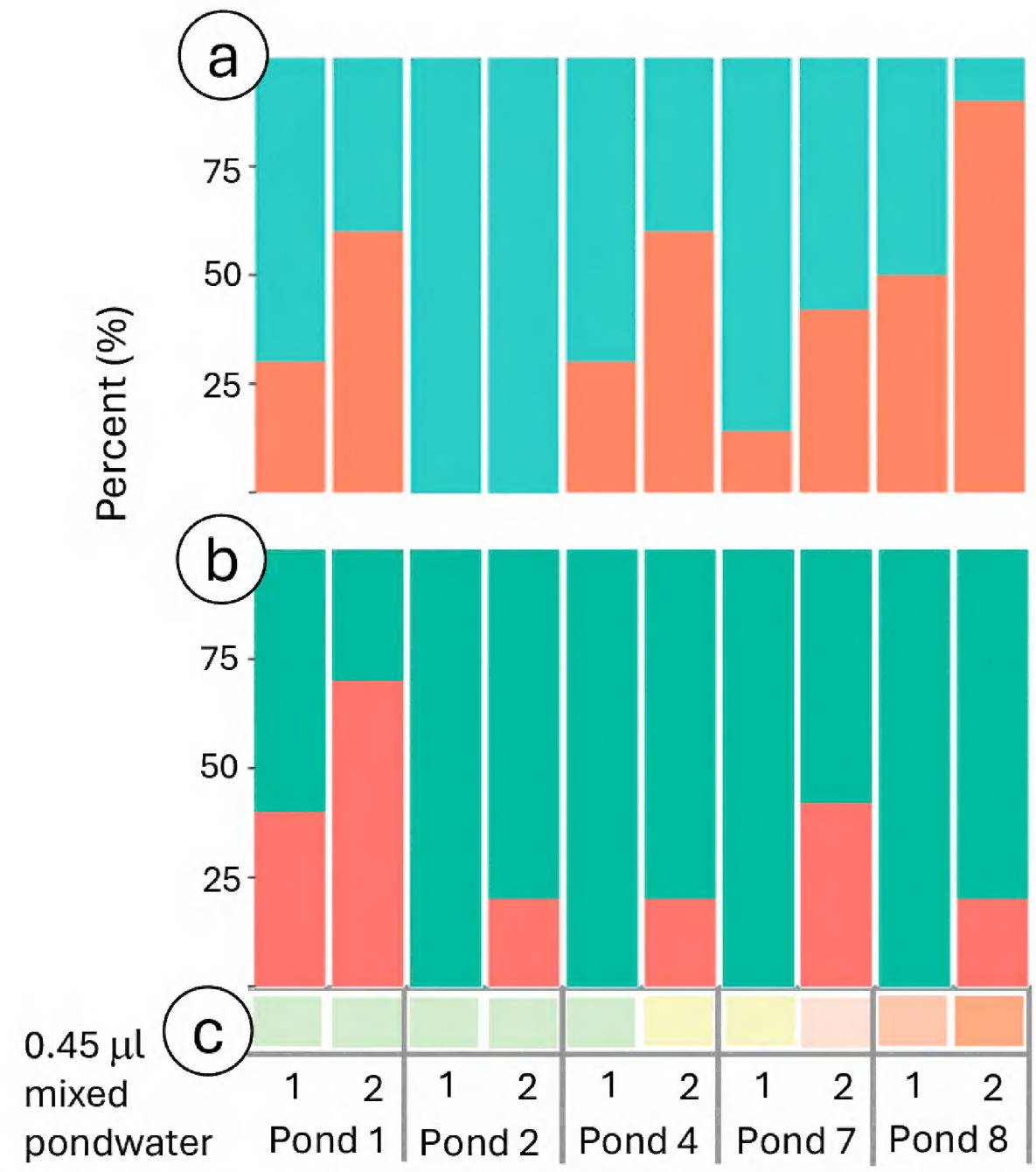

Figure 4. Bd detection from swabs and pond water tested with ddPCR for the five sampled ponds in 2018. **a.** Results (in percentage) from soft skin swabs collected from great crested newts, with positive (orange) and negative (light blue) outcomes; **b.** Same as in (**a**), but for smooth newts; **c.** Results from the mixed pond water filter (0.45 µm), where green = negative Bd results, yellow = one to two positive droplets for one of the replicates, light orange = one replicate with three or more positive droplets, and dark orange = both replicates with three or more droplets.

The DNA concentration in the sample was not found to influence Bd detection in the qPCR reactions (Fig. 5). When comparing the five sample types collected from ponds 6, 7, and 8, the average amount of DNA extracted for each sample type ranged from 33.94 ng, collected with swabs from great crested newts in pond 7, to 2,456 ng of DNA collected from bathwater from smooth newts in pond 6 (Fig. 5). Pond 8 had an overall higher concentration of Bd compared with ponds 6 and 7, where the sample type with the highest concentration had a mean Cq value of 29.9 and was extracted from swabs collected from smooth newts. Mixed pond water had the highest concentration of Bd in the samples collected from pond 8.

Seasonal Bd detection from pond water and newt bathwater in 2024

In 2024, Bd was detected from mixed pond water in four of the ponds at all sampling times, while two ponds had one positive sample at one of the sampling times (ponds 1 and 2; Suppl. material 2: fig. S1). The 2.0 µm filter had slightly fewer Bd-positive qPCRs (77%) compared with the 0.45 µm filter (94%; Suppl. material 2: fig. S2), but this difference was not significant ($t = 0.49$, $p = 0.62$). The amount of DNA input (1 or 5 ng of DNA) produced significantly different results for the

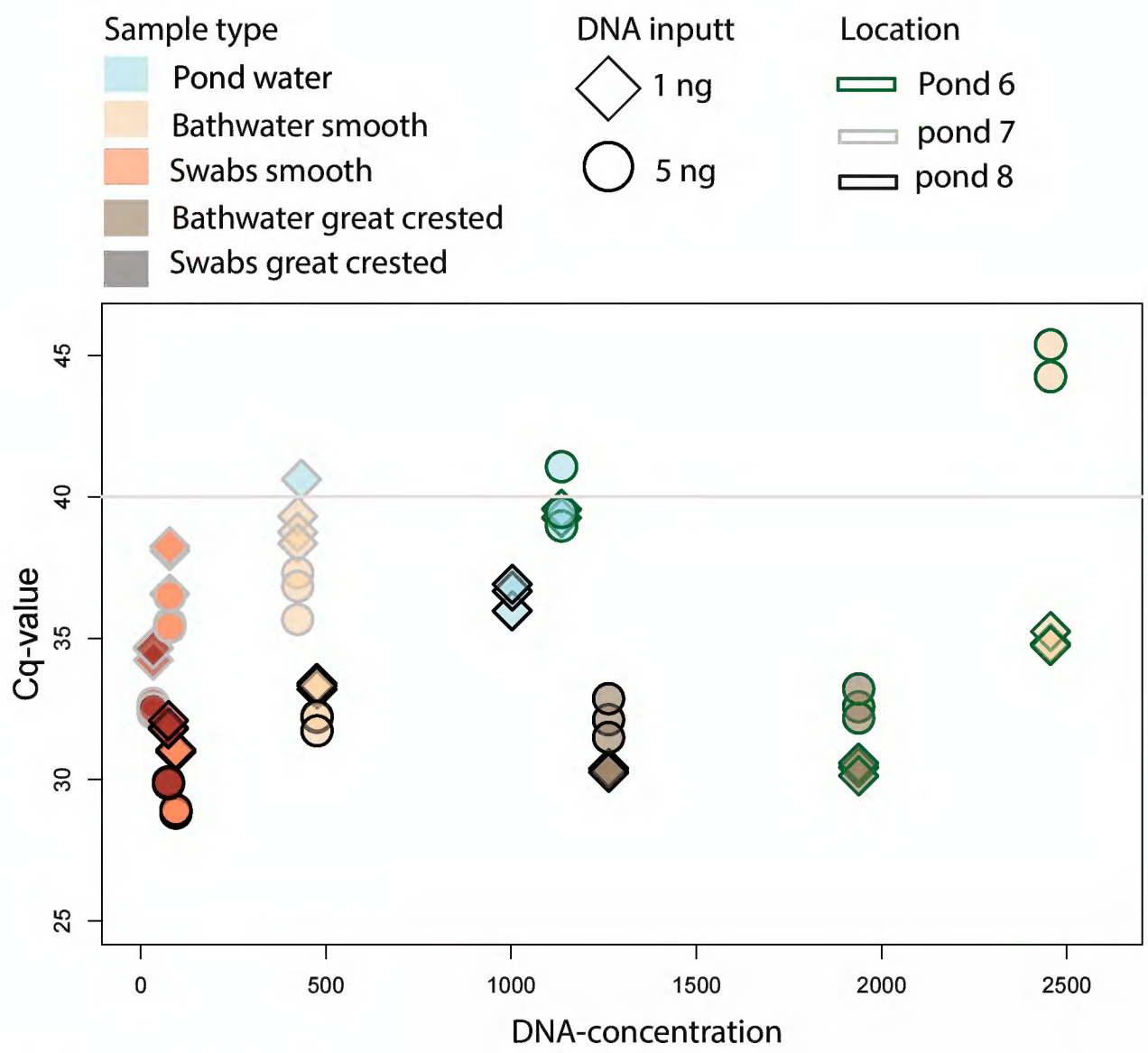

Figure 5. Cq values (duplicates, y-axis) of Bd in relation to the total DNA concentration of the sample (x-axis) from three ponds – pond 6 (dark green border), pond 7 (gray border), and pond 8 (black border) – where the results are divided into the five collected sample types (in blue, brown, coral, and gray) tested with 1 ng (diamond) and 5 ng (circle) of DNA input in the qPCRs.

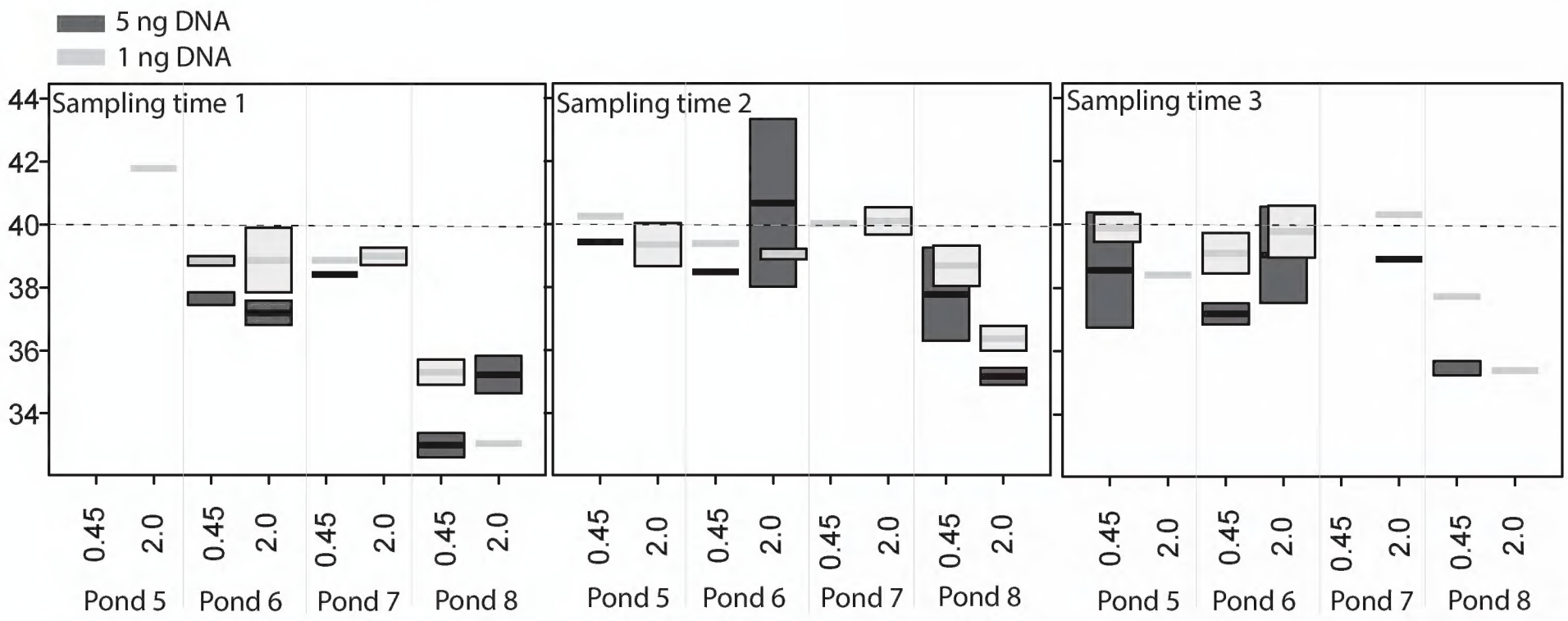

Figure 6. Detection of Bd DNA from pond water sampled at three different times during spring 2024, where two replicated water samples were filtered through either 0.45 μm or 2.0 μm filters and the isolates were tested with both 1 and 5 ng of DNA input in triplicate. Sampling time 1 = May 13–14, sampling time 2 = May 22–23, and sampling time 3 = May 27–28. Data are shown only for ponds 6, 7, and 8, as these were the only ponds with consistent positive findings across all three sampling times. Pond 2 was positive at sampling time 2 (only for the 2.0 μm filter), and pond 1 was positive at sampling time 3 (only for the 0.45 μm filter).

0.45 μm filter, with 5 ng of input yielding a signal at 1.3 fewer cycles compared with 1 ng of input ($t = 2.52$, $p = 0.013$). When pond 8 was analyzed separately, the 2.0 μm filter gave positive signals at 2.04 fewer qPCR cycles compared with the

0.45 μm filter at 1 ng of input ($t = 3.53$, $p < 0.01$) and 1.80 fewer cycles at 5 ng of input ($t = 2.46$, $p = 0.017$), indicating that when successful in Bd detection, the 2.0 μm filter collected higher concentrations of Bd.

Bd detection from toad samples

All sampled toads had a minimum of 52% positive qPCRs for Bd DNA, signifying that all six individuals were positive for Bd (Suppl. material 2: fig. S3). All individuals and qPCRs tested positive for Bd when 5 ng of DNA were used in the reactions for bathwater, soft sandpaper, and swim skin (Fig. 7a, b). Of the 18 qPCRs that were run on 1 ng of DNA extracts from bathwater and soft sandpaper, only one was defined as negative, with qPCR values above 40, whereas seven (38.8%) of the qPCRs from swim skin were false negatives with 1 ng of input DNA (Fig. 7a, b). Results from the soft swabs that were stored dry gave positive Bd results in 9.5% and 50% of the qPCRs with 1 and 5 ng of DNA, respectively, which was similar to the soft swabs stored in buffer, which gave positive signals in 3.5% of the qPCRs with 1 ng of DNA and 48.2% with 5 ng of input DNA (Fig. 7a, b).

The concentration of Bd DNA was highest in the bathwater samples, which on average gave positive signals at 32.2 and 34.3 qPCR cycles with 5 and 1 ng of DNA input, respectively. The bathwater samples were significantly different from soft sandpaper (mean Cq = 33.7 cycles, $p = 0.027$), swim skin (Cq = 33.8 cycles, $p = 0.022$), and soft swabs combined (Cq = 38.6 cycles, $p < 0.01$) at the 5 ng of DNA input. Bd DNA concentrations from bathwater filters and soft sandpaper were not significantly different with 1 ng of DNA input ($p = 0.3$) but were significantly different from swim skin and combined soft swab samples (Fig. 7b).

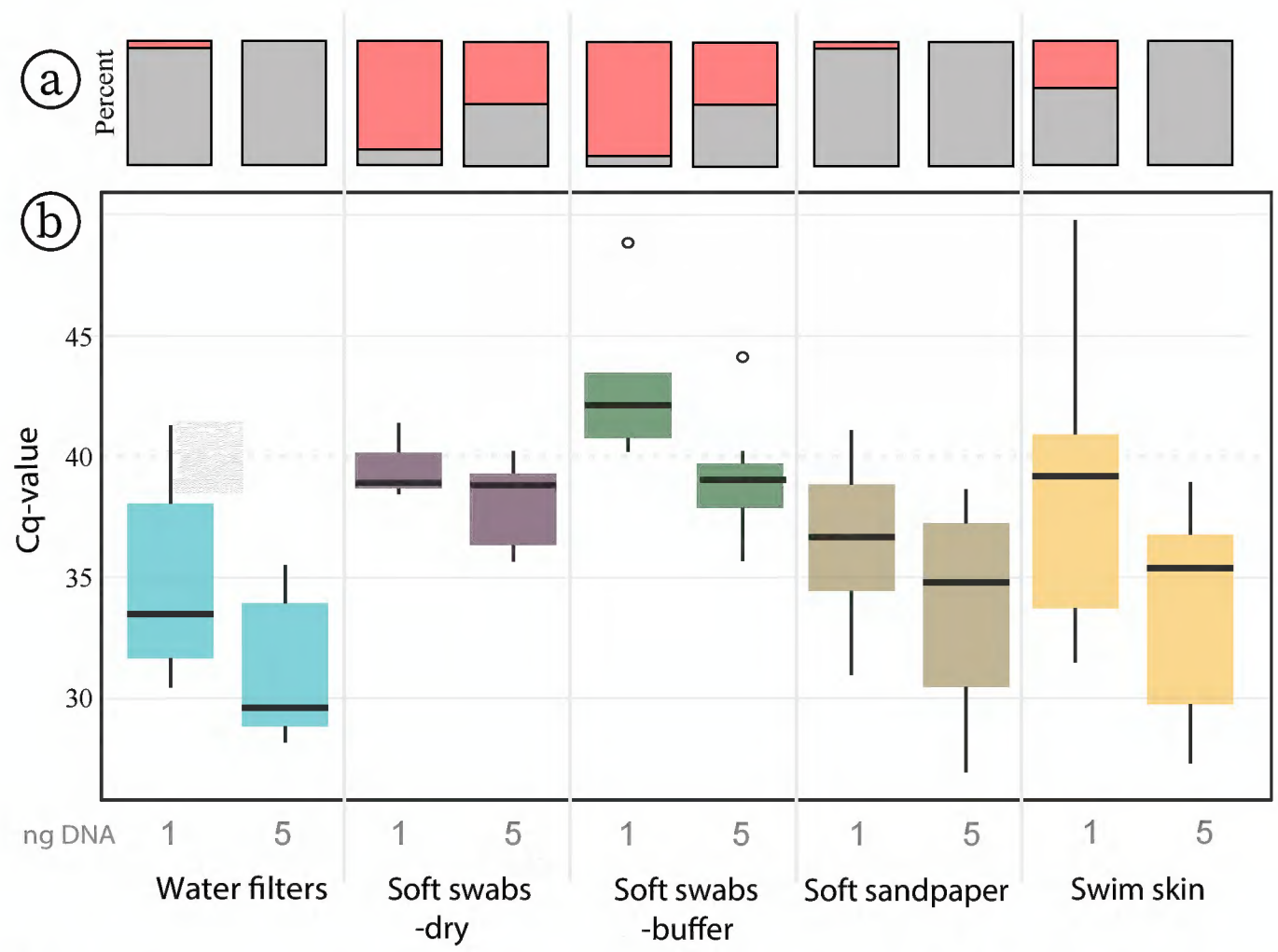

Figure 7. Results from the toad samples. **a.** Percentage of Bd-positive qPCRs (gray) and false negatives (red: all qPCRs between 0.1 and 40.1) for each sample type and amount of DNA input (x-axis in b); **b.** Box plots showing the variation in Cq values for the different sample types after zero values were removed from the dataset. The box plots show the 25–75% quantiles (boxes), the median (black horizontal line), the 95% limits (bars), and outliers (open circles).

Discussion

Amphibians are declining throughout the world, and with invasive diseases being one of the major threats to locally naïve species (Clavero and García-Berthou 2005; Fisher et al. 2012; Pysek et al. 2020), it is vital to have information on local infection status when analyzing population trends over time (Pilliod et al. 2010; West et al. 2024). This study summarizes comparative sampling approaches targeting the parasitic fungus *Batrachochytrium dendrobatidis* from eight newt ponds and one toad population in Norway. In short, combined results showed that the most economical sampling strategy to determine Bd infection status for a pond is to collect local amphibians, place them in water for about 10 minutes, and filter the water. If filtering amphibian bathwater is not an option due to equipment limitations, sampling individuals with soft sandpaper is a straightforward alternative, where results from toads indicate that soft sandpaper produced comparable Bd detection results to bathwater. Since newts are smaller than toads and anurans and have small, delicate fingers, newts should be sampled with extra care. Furthermore, to save funds and time in the laboratory, several individual swabs or soft sandpaper samples can be grouped together and analyzed as a single site sample (Hyatt et al. 2007). If this sample is positive, resources can then be spent on more targeted, in-depth prevalence data across taxa for the identified infected ponds only. If only pond water is to be filtered, the water should be filtered through several filter pore sizes, which can then be extracted individually or in combination before molecular testing. All extracted DNA samples should be tested at a variety of replicated DNA inputs to reduce the chance of inhibition (at lower DNA input levels) and to increase the likelihood of detection (at higher inputs). DNA concentration was not found to correlate with Bd concentration, meaning that even small amounts of extracted DNA had equal chances of detecting Bd across the three compared sample types: water filters, bathwater, and soft skin swabs.

Soft skin swabs yielded inconsistent results across methods and species in this study. For newts, swabbing resulted in five Bd-positive ponds compared with three marginally positive ponds detected by water samples in 2018 (with pond 8 being the only pond with three or more positive droplets in the ddPCR analysis of water samples). The extracts from soft skin swabs also had an overall higher concentration of Bd compared with filtered bathwater samples for both crested newts and smooth newts in 2023. This contrasted with the soft skin swabs collected from the six toads, where about 50–90% of the qPCRs gave false-negative signals at DNA input levels of 5 ng and 1 ng, respectively. The swabbed newts had spent over a month in water before capture, whereas the toads came directly from hibernation when swabbed. Infection prevalence of amphibians has been found to be highest during winter (Garnham et al. 2022), and swabbing before post-hibernation molting, as for the toads, should hence be beneficial for Bd detection. One reason for the large number of negative soft skin swabs from the toads could be a buildup of peptides and other inhibitors in the skin (Le Sage et al. 2021). The swabs from the toads turned brownish from debris – a phenomenon not observed when swabbing wet newts collected directly from ponds. Interestingly, there was little to no effect of inhibitors from the soft sandpaper collected equally from the toads, from which only 0.05% were false negatives with the 1 ng of DNA input, and all tested positive with 5 ng of DNA in the qPCRs (soft sandpaper was not tested on newts). That soft skin swabs are not optimal for Bd testing has also been reported previ-

ously (Garland et al. 2010; Shin et al. 2014; Brannelly et al. 2020b), yet soft skin swabs remain among the most commonly applied sampling methods in external disease testing. The seemingly large potential for false-negative results from soft skin swabs as the collected sample type can impose bias in the results and hence undermine the urgency of conservation or surveillance efforts: if little or no infection is detected, there will be less governmental motivation to spend funds on disease testing in the wild. This is probably even more relevant for countries that have not experienced observable mass mortality of species (Scheele et al. 2019), such as most European countries, but where Bd can still cause sublethal fitness effects through reduced growth (Bielby et al. 2015; Burrow et al. 2017), reduced genetic variability (Cortazar-Chinarro et al. 2022), alternative gene expression patterns (Eskew et al. 2018; Chondrelli et al. 2024), and individual mortality (Meurling et al. 2024). In addition, since long-term monitoring datasets of amphibian populations in general remain scarce (Nyström et al. 2007; Taugbøl and Sandaas 2025), wild populations with and without infection should be monitored even without evidence of mass mortality (Pilliod et al. 2010; West et al. 2024). By only changing the swabbing method from soft skin swabs to soft sandpaper, the results from the toad samples indicate an almost 100% detection rate of Bd.

Filtered pond water extracts detected Bd DNA less frequently than bathwater and soft skin swabs collected from newt ponds in this study. Environmental DNA is a great tool for species detection (Deiner et al. 2017; Ruppert et al. 2019; Pawlowski et al. 2021), but variations in sampling methodologies affect the obtained results – for instance, differences between filter pore sizes (Snyder et al. 2023; Jo 2024; Janik-Superson et al. 2025; Taugbøl et al. 2025), sampling depth (Fukumori et al. 2024; Taugbøl et al. 2025), sampling time during the day (Macher et al. 2024), and season (Wacker et al. 2019; Congram et al. 2022). Sampling in the wild must therefore be optimized for the organism(s) of interest to reduce the chance of false-negative results, which can occur even when the targeted organism(s) is clearly present in the environment (Pinfield et al. 2019; Taugbøl et al. 2025). The likelihood of Bd detection from a water sample will depend on several interactive factors, such as the number of hosts present, infection prevalence and virulence (host skin sloughing, which releases Bd DNA into the water), and the rate of zoospore release from Bd sporangia during the sampling time. If the location (here, pond) is small and infected hosts are plentiful, a water sample will likely be positive, as seen for pond 8 in this study. If the location is large, contains few amphibians with low infection loads, or when water sampling does not coincide with host presence, the likelihood of obtaining a positive water sample is much lower – as pond 2 could exemplify in this study. Pond 2 has a small population of newts, probably due to a combination of artificially alternating water levels for crop irrigation and a healthy population of grass snakes that feed on newts (Gregory and Isaac 2004). Historically, pond 2 tested positive for Bd in 2017 (Taugbøl et al. 2021) and weakly positive in 2018 (Fig. 3). It remained negative for five consecutive years, then returned a weakly positive result in 2023. In 2024, only one out of twelve water samples tested positive – all six qPCRs on that sample (from the 2.0 μm filter) detected Bd. The remaining eleven water samples, as well as the bathwater sample from the same pond, tested negative.

In this study, Bd detection probabilities in pond water changed only slightly with different filter pore sizes, even though the larger-pore filter processed more than twice as much water. In general, larger volumes of filtered water have been

shown to increase the likelihood of detecting eDNA from rare species (Fossøy et al. 2020; Schabacker et al. 2020). However, further testing of different filter pore sizes is needed. For example, results from pond 8 showed a higher number of positive qPCRs from the 0.45 µm filter, although these samples had, on average, lower Bd concentrations compared with those from the 2.0 µm filter. The smaller 0.45 µm filter was also shown to capture more newt eDNA in a previous study (Taugbøl et al. 2025). Filters with both smaller and larger pore sizes than those tested here should also be considered. eDNA particle sizes for Daphnia magna were found to predominate in the 0.22 µm filter (Moushomi et al. 2019), and sequential filtering of water through 0.45 µm and 0.22 µm filters showed that about 30% of diversity was uniquely obtained from the second, smaller filter porosity (Janik-Superson et al. 2025). In contrast, other studies have shown larger pore-sized filters to obtain higher DNA concentrations, as for fish eDNA (Barnes et al. 2021; Jo 2024).

Bd detection varied somewhat with seasonal sampling time in 2024, as was also observed for one pond in 2017 (pond 4; Taugbøl et al. 2021). Several other studies have similarly reported inconsistent Bd detection with season, with 29% reported overlap between positive Bd sites in southern Ontario, Canada, for sites first sampled in May and then resampled in July (Congram et al. 2022). Other findings on Bd detection in the wild include temporal heterogeneity in Bd concentration within positive sites, with higher detected concentrations from water samples in spring and fall compared with summer (Chestnut et al. 2014). In the present study, ponds 7 and 8 had the highest Bd concentrations during the first sampling period in 2024, but the two later sampling times detected Bd in one additional pond per sampling time, and the concentration of Bd in pond 6 also increased with time (Fig. 6). Pond 1 and pond 2 have cooler spring temperatures due to more northern locations and slightly higher elevations. Bd is known to grow well in cultures kept at cold temperatures and can also withstand periods of freezing, but optimal temperatures for growth are reported to range between 17 °C and 25 °C (Voyles et al. 2012). The increased detection with time may reflect higher water temperatures and more sporulation of Bd zoospores. However, the three sampling points included in this study – spanning only two weeks between the first and third – do not provide sufficient temporal resolution to statistically account for local seasonal variation, especially without pond-specific temperature data. Longer seasonal studies from infected ponds are needed to determine how temperature affects Bd eDNA in water and whether temperature influences the host animals' behavior differently when infected, such as by prompting earlier departure from the pond, which again can affect the amount of Bd DNA in the water.

Monitoring wild populations and collecting background data to understand natural population fluctuations are more important than ever. Pre-decline population data are rarely available, and the causes of decline are typically complex and interrelated (Nyström et al. 2007; Taugbøl and Sandaas 2025). Given the seemingly unpredictable Bd prevalence at individual ponds and between years, the high natural variability in amphibian population sizes (Pechmann et al. 1991), and the low number of newt ponds included in this study, statistical comparisons of population sizes between infected and uninfected ponds were not conducted. However, by looking at the newt capture data separately for each pond (Fig. 3), there are no clear indications of any major population declines attributable to Bd. If anything, the number of smooth newts has been increasing in most ponds during the last five years. The number of crested newts has been low to sporadic across all ponds

and sampling years, except for pond 8, which has maintained a stable population throughout the sampling period. The crested newt population in pond 8 reached its lowest point in 2016, likely due to natural factors such as the harsh winter of 2014, which may have reduced juvenile survival. However, even if ongoing Bd infection is not causing short-term declines in the Norwegian newt species, Bd presence – linked to climate change and evolving infection intensity – could tell a different story in the future.

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Additional information

Conflict of interest

The author has declared that no competing interests exist.

Ethical statement

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Use of AI

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Author contributions

Conceptualization: AT. Data curation: AT. Formal analysis: AT. Funding acquisition: AT. Visualization: AT. Writing – original draft: AT.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

All sampling presented in this study was approved by the Norwegian Environment Agency (ref. 2012/3515, 2014/2025, 2017/1082, 2021/1876, 2023/5208 ART-VI-ID).

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Supplementary material 1

Data for the sampling years 2018, 2023 and 2024

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Data type: xlsx

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Supplementary material 2

Supplementary figures

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Data type: docx

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